

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner  
 US Department of Commerce  
 United States Patent and Trademark  
 Office, PCT  
 2011 South Clark Place Room  
 CP2/5C24  
 Arlington, VA 22202  
 ETATS-UNIS D'AMERIQUE  
 in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 04 April 2001 (04.04.01)	
<b>International application No.</b> PCT/US00/16559	<b>Applicant's or agent's file reference</b> 5986/2F684-WO
<b>International filing date (day/month/year)</b> 15 June 2000 (15.06.00)	<b>Priority date (day/month/year)</b> 16 June 1999 (16.06.99)
<b>Applicant</b> DEVI, Lakshmi, A. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:16 January 2001 (16.01.01)☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<b>The International Bureau of WIPO</b> 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	<b>Authorized officer</b>  S. Mafla Telephone No.: (41-22) 338.83.38
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# P A T COOPERATION TREATY

*HR*

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

## PCT

WRITTEN OPINION

(PCT Rule 66)

<b>To:</b> PAUL FEHLNER DARBY & DARBY P.C. 805 THIRD AVENUE NEW YORK NY 10022-7513
<b>DUE:</b> <u>September 20, 2001</u>
Docketed on <u>7/26</u> by <u>DP</u> for
Docketed without file <input type="checkbox"/>
Attorney <u>PFE</u>

Date of Mailing (day/month/year)	<b>20 JUL 2001</b>
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Applicant's or agent's file reference 5986/2F684-W		<b>REPLY DUE</b> within <b>TWO</b> months from the above date of mailing
International application No. PCT/US00/16559	International filing date (day/month/year) 15 JUNE 2000	Priority date (day/month/year) 16 JUNE 1999
International Patent Classification (IPC) or both national classification and IPC Please See Supplemental Sheet.		
Applicant NEW YORK UNIVERSITY		

1. This written opinion is the <u>first</u> (first, etc.) drawn by this International Preliminary Examining Authority.	
2. This opinion contains indications relating to the following items:	
I	<input checked="" type="checkbox"/> Basis of the opinion
II	<input type="checkbox"/> Priority
III	<input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
IV	<input type="checkbox"/> Lack of unity of invention
V	<input checked="" type="checkbox"/> Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	<input type="checkbox"/> Certain documents cited
VII	<input type="checkbox"/> Certain defects in the international application
VIII	<input type="checkbox"/> Certain observations on the international application
3. The applicant is hereby invited to reply to this opinion.	
<b>When?</b>	See the time limit indicated above. <del>The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).</del>
<b>How?</b>	By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.
<b>Also</b>	For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.
If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.	
4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: <u>16 OCTOBER 2001</u>	

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ROBERT LANDSMAN <i>Robert Landsman</i>
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

## I. Basis of the opinion

## 1. With regard to the elements of the international application:\*

☒ the international application as originally filed☒ the description:

pages 1-65

, as originally filed

pages NONE

, filed with the demand

pages NONE

, filed with the letter of

☒ the claims:

pages 66-70

, as originally filed

pages NONE

, as amended (together with any statement) under Article 19

pages NONE

, filed with the demand

pages NONE

, filed with the letter of

☒ the drawings:

pages 1-7

, as originally filed

pages NONE

, filed with the demand

pages NONE

, filed with the letter of

☒ the sequence listing part of the description:

pages NONE

, as originally filed

pages NONE

, filed with the demand

pages NONE

, filed with the letter of

## 2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language \_\_\_\_\_ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

## 3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

☐ contained in the international application in printed form.☐ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☒ The amendments have resulted in the cancellation of:☒ the description, pages NONE☒ the claims, Nos. NONE☒ the drawings, sheets/fig NONE5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 24-32

because:

☐ the said international application, or the said claim Nos. \_ relate to the following subject matter which does not require international preliminary examination (*specify*).

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. \_ are so unclear that no meaningful opinion could be formed (*specify*).

☐ the claims, or said claims Nos. \_ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 24-32.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. statement**

Novelty (N)	Claims	<u>1-23</u>	YES
	Claims	<u>NONE</u>	NO
Inventive Step (IS)	Claims	<u>NONE</u>	YES
	Claims	<u>1-23</u>	NO
Industrial Applicability (IA)	Claims	<u>1-23</u>	YES
	Claims	<u>NONE</u>	NO

**2. citations and explanations**

Claims 1-23 possess novelty under PCT Article 33(2) since no prior art reference discloses specifically teach an isolated heterodimeric receptor which comprises an opioid receptor subunit and a GPCR subunit.

Claims 1, 2, 6-12 and 16-18 lack an inventive step under PCT Article 33(3) as being obvious over Cvejic et al. in view of Knapp et al. and further in view of Sambrook et al. Cvejic et al. teach tagged opioid receptors and that the delta-opioid receptor does dimerize with either delta- or mu-opioid receptors (Abstract; p. 26959, right column, first full paragraph), showing that these receptors are endogenously expressed in the same cell type. In addition, it is well-known in the art that opioid receptors are endogenously expressed in neuronal cells, neuronal cells all being one type of cell. Furthermore, one of ordinary skill in the art would expect certain types of cells to express various opioid receptor types. Cvejic et al. also teach screening for modulators of these receptors with regard to trafficking of these receptors as measured by internalization. Cvejic et al. do not teach a recombinant host cell that expresses a functional heterodimeric opioid receptor. However, Knapp et al. do teach various types of isolated, cloned opioid receptors as well as screening assays using radioligand binding techniques and discuss that these receptors act via cAMP. It would have been obvious to one of ordinary skill in the art at the time of the invention to have co-transfected host cells with the DNA of Knapp et al. in order to have co-expressed these receptors in a cell, which would then be expected to dimerize. Neither Cvejic et al. nor Knapp et al. teach a method of transfecting host cells. However, Sambrook et al. do teach a method of transfecting host cells. It would have been obvious to one of ordinary skill in the art at the time of the invention to have substituted the DNA of Knapp et al. for the DNA in the polycloning region of Sambrook et al. for the purposes of transfecting a cell in order to characterize the protein.

Claims 1, 3, 6, 7, 9-11, 13, 16, 17, 19 and 21-23 lack an inventive step under PCT Article 33(3) as being obvious over Ng (Continued on Supplemental Sheet.)

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

**TIME LIMIT:**

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

**CLASSIFICATION:**

The International Patent Classification (IPC) and/or the National classification are as listed below:  
IPC(7): C12P 21/06, 21/04; C12N 1/20, 15/00, 15/09, 15/63, 15/70, 15/74, 5/00, 5/02; C07K 1/00, 14/00, 17/00; C07H 21/04; G01N 33/53, 33/567 and US Cl.: 435/7.1, 7.2, 69.1, 70.1, 71.1, 71.2, 252.3, 325, 471; 530/350; 536/23.5

**V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):**

et al. in view of Civelli et al. and further in view of Knapp et al. Ng et al. teach tagged dopamine receptors (second paragraph under "Materials and Methods") and that dopamine receptors form dimers. In addition, it is well-known in the art that opioid and dopamine receptors are endogenously expressed in neuronal cells, neuronal cells all being one type of cell. Furthermore, one of ordinary skill in the art would expect certain types of cells to express both opioid and dopamine receptors. Both Civelli and Ng et al. teach isolated dopamine receptors. Ng et al. also teach the transfection of dopamine receptors into host cells. Neither Civelli et al. or Ng et al. teach opioid receptors. However, Knapp et al. do teach various types of isolated, cloned opioid receptors as well as screening assays using radioligand binding techniques and discuss that these receptors act via cAMP. Ng et al. and Civelli et al. also teach screening methods measuring cAMP and binding affinity for dopamine receptors. It would have been obvious to one of ordinary skill in the art at the time of the invention to have co-transfected host cells with the DNA of Knapp et al. in order to have co-expressed these receptors in a cell, which would then be expected to dimerize.

Claims 1, 4, 6, 7, 9-11, 14, 16, 17, 19 and 21-23 lack an inventive step under PCT Article 33(3) as being obvious over Frielle et al. in view of Hebert et al. and further in view of Knapp et al. Hebert et al. teach tagged adrenergic receptors (first paragraph under "Experimental Procedures") and that adrenergic receptors form dimers. In addition, it is well-known in the art that opioid and adrenergic receptors are endogenously expressed in neuronal cells, neuronal cells all being one type of cell. Furthermore, one of ordinary skill in the art would expect certain types of cells to express both opioid and adrenergic receptors. Both Frielle et al. and Hebert et al. teach isolated adrenergic receptors. and both teach the transfection of adrenergic receptors into host cells. Neither Frielle et al. or Hebert et al. teach opioid receptors. However, Knapp et al. do teach various types of isolated, cloned opioid receptors as well as screening assays using radioligand binding techniques and discuss that these receptors act via cAMP. Frielle et al. and Hebert et al. also teach screening methods measuring cAMP and binding affinity for adrenergic receptors. It would have been obvious to one of ordinary skill in the art at the time of the invention to have co-transfected host cells with the DNA of Knapp et al. in order to have co-expressed these receptors in a cell, which would then be expected to dimerize.

Claims 1, 5, 7, 9-11, 15, 17, 19 and 21-23 lack an inventive step under PCT Article 33(3) as being obvious over Power et al. in view of Rodriguez-Frade et al. and further in view of Knapp et al. Rodriguez-Frade et al. teach tagged chemokine receptors (Abstract) and that chemokine receptors form dimers. In addition, it is well-known in the art that opioid and chemokine receptors are endogenously expressed in neuronal cells, neuronal cells all being one type of cell. Furthermore, one of ordinary skill in the art would expect certain types of cells to express both opioid and chemokine receptors. Both Power et al. and Rodriguez-Frade et al. teach isolated chemokine receptors and the transfection of dopamine receptors into host cells. Neither Power et al. or Rodriguez-Frade et al. teach opioid receptors. However, Knapp et al. do teach various types of isolated, cloned opioid receptors as well as screening assays using radioligand binding techniques and discuss that these receptors act via cAMP. It would have been obvious to one of ordinary skill in the art at the time of the invention to have co-transfected host cells with the DNA of Knapp et al. in order to have co-expressed these receptors in a cell, which would then be expected to dimerize.

Claims 1-23 possess industrial applicability under PCT Article 33(4) because the compounds and methods taught by the specification encompassed by the claims can be utilized in the field for which they were intended.

----- NEW CITATIONS -----

NONE

WRITTEN OPINION

International Application No.

PCT/US00/16559

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient).

Continuation of: Boxes I - VIII

Sheet 11

# PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCH AUTHORITY

PCT

To: PAUL FEHLNER  
DARBY & DARBY P.C.  
805 THIRD AVENUE  
NEW YORK NY 10022-7513

## NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

**DUE:** December 5, 2000  
Docketed on 10/12 by DP or  
Docketed without file ☒

Attorney \_\_\_\_\_

Date of Mailing  
(day/month/year)

**05 OCT 2000**

Applicant's or agent's file reference  
5986/2F684-W

**FOR FURTHER ACTION** See paragraphs 1 and 4 below

International application No.  
PCT/US00/16559

International filing date  
(day/month/year)  
15 JUNE 2000

Applicant  
NEW YORK UNIVERSITY

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.  
☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer  
ROBERT LANDSMAN

Telephone No. (703) 308-0196

# P A T COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

TO: LMG  
ATTY REVIEWED LMG  
DATE: 11/28/01

## PCT

### NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

To: PAUL FEHLNER DARBY & DARBY P.C. 805 THIRD AVENUE NEW YORK NY 10022-7513
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Date of Mailing (day/month/year)	06 NOV 2001
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Applicant's or agent's file reference 5986/2F684-W			IMPORTANT NOTIFICATION		
International application No. PCT/US00/16559	International filing date (day/month/year) 15 JUNE 2000	Priority Date (day/month/year) 16 JUNE 1999			
Applicant NEW YORK UNIVERSITY					

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ROBERT LANDSMAN Telephone No. (703) 308-0196
Facsimile No. (703) 305-3230	

# PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: PAUL FEHLNER  
DARBY & DARBY P.C.  
805 THIRD AVENUE  
NEW YORK NY 10022-7513

## PCT

### NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 5986/2F684-W	Date of Mailing (day/month/year) <span style="float: right; font-size: 1.2em;">05 OCT 2000</span>
International application No. PCT/US00/16559	International filing date (day/month/year) 15 JUNE 2000
Applicant NEW YORK UNIVERSITY	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231  Facsimile No. (703) 305-3230	Authorized officer ROBERT LANDSMAN  Telephone No. (703) 308-0196
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## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 5986/2F684-W	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/16559	International filing date (day/month/year) 15 JUNE 2000	(Earliest) Priority Date (day/month/year) 16 JUNE 1999
Applicant NEW YORK UNIVERSITY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
- ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:
- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☐ Certain claims were found unsearchable (See Box I).
3. ☒ Unity of invention is lacking (See Box II).
4. With regard to the title,
- ☒ the text is approved as submitted by the applicant.
- ☐ the text has been established by this Authority to read as follows:
5. With regard to the abstract,
- ☒ the text is approved as submitted by the applicant.
- ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the drawings to be published with the abstract is Figure No. \_\_\_\_\_
- ☐ as suggested by the applicant.
- ☐ because the applicant failed to suggest a figure.
- ☐ because this figure better characterizes the invention.
- ☒ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT /16559

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-23

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US/01/16559

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.2, 69.1, 70.1, 71.1, 71.2, 252.3, 325, 471; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, USPATFULL

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KNAPP et al. Molecular biology and pharmacology of cloned opioid receptors. FASEB J. April 1995, Vol. 9, pages 516-525, see entire document, especially pages 516-522 and Figures 4 and 5.	1-19, 21-23
Y	SAMBROOK et al. Molecular Cloning; a laboratory manual. 2nd ed. Cold Spring Harbor Press, 1989, pages 17.2-17.44, see entire document.	11-18
Y	CVEJIC et al. Dimerization of the delta-opioid receptor: implication for a role in receptor internalization. J. Biol. Chem., Vol. 272.No. 43, pages 26959-26964, 1997, see entire document.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*Q* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

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Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ROBERT LANDSMAN

Telephone No. (703) 308-0196

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CIVELLI et al. Molecular Biology of the dopamine receptors. Eur. J. Pharmacol. 1991, Vol. 207, pages 277-286, especially pages 278-280.	1,3,6,7,9- 11,13,16,17,19,21 -23
Y	NG, GYK et al. Dopamine D2 receptor dimers and receptor-blocking peptides. Biochem. Biophys. Res. Comm. 1996, Vol. 227, pages 200-204.	1,3,6,7,9- 11,13,16,17,19,21 -23
Y	FRIELLE et al. Properties of the B1- and B2-adrenergic receptor subtypes revealed by molecular cloning. Clin. Chem. 1989, Vol. 35, No. 5, pages 721-725, see entire document.	1,4,6,7,9-1 1,14,16,17, 19,21
Y	HEBERT et al. A peptide derived from a b2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. 1996, Vol. 271. No.27, pages 16384-16392, see entire document.	1,4,6,7,9- 11,14,16,17,19,22, 23
Y	POWER et al. Cloning and characterization of human chemokine receptors. Trends in Pharmacol. Sci. June 1996, Vol 17, pages 209-213, especially Figure 2 and page 212.	1,5,7,9- 11,15,17,19,22
Y	RODRIGUEZ-FRADE et al. The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. March 1999, Vol. 96, pages 3628-3633, see entire document, especially Figure 1 and Figure 5.	1,5,7,9- 11,15,17,19,22,23

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C12P 21/06, 21/04; C12N 1/20, 15/00, 15/09, 15/63, 15/70, 15/74, 5/00, 5/02; C07K 1/00, 14/00, 17/00; C07H 21/04; G01N 33/53, 33/567

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/7.1, 7.2, 69.1, 70.1, 71.1, 71.2, 252.3, 325, 471; 530/350; 536/23.5

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claim(s) 1-23, drawn to an isolated heterodimeric receptor, a recombinant host cell and a method of screening for a compound.

Group II, claim(s) 24-31, drawn to a bispecific, bivalent compound and a pharmaceutical composition.

Group III, claim 32, drawn to a method of treating a disease.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of Group I is a heterodimeric receptor. The special technical feature of Group II is a bispecific, bivalent compound. The special technical feature of Group III is a method of treating a disease. The special technical feature of each Group is not the same or does not correspond to the special technical feature of any other Group. The products of Groups I and II are structurally and functionally distinct, and the methods of Groups I and III require different method steps and reagents for achieving different goals. The Groups are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

CHAPTER I  
PCT TELEPHONE MEMORANDUM  
FO  
LACK OF UNITY OF INVENTION

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PCT No.: PCT/US00/16559

Examiner: ROBERT LANDSMAN

Attorney spoken to: PAUL FEHLNER

Date of call: 11 SEPTEMBER 2000

- ☐ Amount of payment approved:
- ☐ Deposit account number to be charged:
- ☐ Attorney elected to pay for ALL additional inventions
- ☐ Attorney elected to pay only for the additional inventions covered by
  - ☐ Group(s):
  - encompassing --
  - ☐ Claim(s):
- ☒ Attorney elected **NOT** to pay for any additional inventions, therefore, only the first claimed invention (Group I) covered by Claim(s) 1-23 has been searched.
- ☒ Attorney was orally advised that there is no right to protest for any group not paid for.
- ☒ Attorney was orally advised that any protest must be filed no later than 15 days from the mailing of the Search Report (PCT/ISA/210).

**Time Limit For Filing A Protest**

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2, applicant may protest the holding of lack of unity only with respect to the group(s) paid for.

**Detailed Reasons For Holding Lack Of Unity Of Invention:**

**Detailed Reasons For Holding Lack of Unity Of Invention:**  
(Continued on a separate sheet)

**Note: A copy of this form must be attached to the Search Report.**

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ATTACHMENT TO CHAPTER I PCT TELEPHONE MEMORANDUM  
FOR  
LACK OF UNITY OF INVENTION

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Group I, claim(s) 1-23, drawn to an isolated heterodimeric receptor, a recombinant host cell and a method of screening for a compound.

Group II, claim(s) 24-31, drawn to a bispecific, bivalent compound and a pharmaceutical composition.

Group III, claim 32, drawn to a method of treating a disease.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of Group I is a heterodimeric receptor. The special technical feature of Group II is a bispecific, bivalent compound. The special technical feature of Group III is a method of treating a disease. The special technical feature of each Group is not the same or does not correspond to the special technical feature of any other Group. The products of Groups I and II are structurally and functionally distinct, and the methods of Groups I and III require different method steps and reagents for achieving different goals. The Groups are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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(US). JORDAN, Bryen, A. [US/US]: 243 East 33rd Street,  
New York, NY 10016 (US).

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(74) Agents: FEHLNER, Paul, F. et al.: Darby & Darby, P.C.,  
805 Third Avenue, New York, NY 10022-7513 (US).

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(71) Applicant (*for all designated States except US*): NEW  
YORK UNIVERSITY [US/US]: 4 Washington Square,  
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(72) Inventors; and

(75) Inventors/Applicants (*for US only*): DEVI, Lakshmi, A.  
[IN/US]: 9 Glenwood Avenue, New Rochelle, NY 10801

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 00/77238 A1

(54) Title: HETERODIMERIC OPIOID G-PROTEIN COUPLED RECEPTORS

(57) Abstract: Opioid receptors form functional heterodimers with each other and with other G-protein coupled receptors, such as dopamine receptors, adrenergic receptors, or chemokine receptors. These receptors can be exploited for high throughput screening of compounds to identify heterodimer opioid receptor modulators (agonists and antagonists). The invention also relates to identification of novel heterodimer receptor ligands and synergistic compositions, which can provide strategies for analgesia, narcotic addiction, hypertension, HIV infection, and immune system function.

## 5                   **HETERODIMERIC OPIOID G-PROTEIN COUPLED RECEPTORS**

The research leading to the present invention was supported, in part, by National Institute of Health Grants DA 08863 (National Institute of Drug Abuse) and NS1788 (National Institute of Neurological Diseases and Stroke). Thus, the United  
10       States Government has certain rights in the invention.

### **FIELD OF THE INVENTION**

The present invention relates generally to opioid receptors, and more specifically to heterodimer opioid receptor, as well as to methods for identifying  
15       modulators (agonists and antagonists) of such receptors. The invention also relates to identification of novel heterodimer receptor ligands and synergistic compositions, which can provide strategies for analgesia, narcotic addiction, hypertension, and immune system function.

### 20                   **BACKGROUND OF THE INVENTION**

The opioid system modulates several physiological processes including analgesia, stress response, immune response, and neuroendocrine function (Herz, *Opioids* Vol. 1, Springer-Verlag, Berlin, 1993). Pharmacological and molecular cloning studies have identified three opioid receptor types, delta ( $\delta$ ), kappa ( $\kappa$ ), and  
25       mu ( $\mu$ ), that mediate these diverse effects (Miotto *et al.*, *The Pharmacology of Opioid Peptides*, L. Tseng ed., 57-71, Harwood Acad. Publishers, 1995; Kieffer *et al.*, *Cell Mol. Neurobiol.*, 15:615-35, 1995). The opioid receptors are known to couple with pertussis toxin sensitive G-proteins.

Little is known, however, about the ability of these receptors to interact  
30       to form new functional structures, the simplest of which would be a dimer. Structural and biochemical studies reveal that other G-protein coupled receptors (GPCRs) interact to form homodimers (Herbert and Bouvier, *Biochem Cell Biol.*, 76:1-11,

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1998; Gouldson *et al.*, Protein Eng., 11:1181-93, 1998). Moreover, non-functional GABA receptors heterodimerize to form a functional receptor, suggesting that dimerization is crucial for this receptor function (Jones *et al.*, Nature, 396:674-679, 1998; Kaupmann, *et al.*, Nature, 396:683-687, 1998; White *et al.*, Nature, 396:679-682, 1998; and Kuner *et al.*, Science, 283:74-77, 1999).

It is now clear from work carried out in many laboratories over the last twenty years that there are three well-defined or "classical" types of opioid receptors: mu ( $\mu$ ), delta ( $\delta$ ), and kappa ( $\kappa$ ). Genes encoding these receptors have been cloned (Evans *et al.*, Science, 258:1952, 1992; Kieffer *et al.*, Proc. Natl. Acad. Sci. USA, 89:12048, 1992; Chen *et al.*, Mol. Pharmacol., 44:8, 1993; Minami *et al.*, FEBS Lett., 329:291, 1993). More recently, cDNA was identified encoding an "orphan" receptor that has a high degree of homology to the "classical" opioid receptors; on the basis of its structural homology, this receptor has been classified as an opioid receptor and has been named ORL (opioid receptor-like) (Mollereau *et al.*, FEBS Lett., 341:33, 1994). As would be predicted from their known abilities to couple through pertussis toxin-sensitive G-proteins, all of the cloned opioid receptors possess the same general structure of an extracellular N-terminal region, seven transmembrane domains and intracellular C-terminal tail structure. There is pharmacological evidence that subtypes of each receptor exist. Other types of novel, less well-characterized opioid receptors (termed  $\epsilon$ ,  $\gamma$ ,  $\iota$ ,  $\zeta$ ) have also been postulated. The  $\sigma$ -receptor, however, is no longer regarded as an opioid receptor.

Opioid receptors are reviewed extensively in a publication entitled "Opioid" edited by A. Herz and in a publication from Tocris Cookson Inc. (USA)/Tocris Cookson Ltd. (UK) entitled "Opioid Receptors", co-authored by A. Corbett, S. McKnight and G. Henderson, 1999.

### $\mu$ -Receptor Subtypes

The MOR-1 gene, encoding for one form of the  $\mu$ -receptor, shows approximately 50-70% homology to the genes encoding for the  $\delta$ -(DOR-1),  $\kappa$ -(KOR-1) and orphan (ORL<sub>1</sub>) receptors. Two splice variants of the MOR-1 gene

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have been cloned, differing only in the presence or absence of 8 amino acids in the C-terminal tail. The splice variants exhibit differences in their rate of onset and recovery from agonist-induced internalization but their pharmacology does not appear to differ in ligand binding assays (Koch *et al.*, N.S. Archives of Pharmacology, 5 357:SS44, 1998). Furthermore, in the MOR-1 knockout mouse, morphine does not induce antinociception, demonstrating that at least in this species morphine-induced analgesia is not mediated through  $\delta$ - or  $\kappa$ -receptors (Matthes *et al.*, Nature, 383:818, 1996). Similarly morphine does not exhibit positive reinforcing properties or an ability to induce physical dependence in the absence of the MOR-1 gene. The  $\mu_1/\mu_2$  10 subdivision was proposed by Pasternak and colleagues to explain their observations, made in radioligand binding studies, that  $^3\text{H}$ -labelled- $\mu$ ,  $\delta$ , and  $\kappa$  ligands displayed biphasic binding characteristics (Wolozin and Pasternak, Proc. Natl. Acad. Sci. USA, 78:6181, 1981).

Several related observations suggest the existence of a yet unidentified 15 form of  $\mu$ -receptor of which analogues of morphine with substitutions at the 6 position (*e.g.*, morphine-6 $\beta$ -glucuronide, heroin and 6-acetyl morphine) are agonists, but with which unsubstituted morphine itself does not interact (Rossi *et al.*, Neuroscience Letters, 216:1, 1996).

#### $\delta$ -Receptor Subtypes

20 The DOR-1 gene is the only  $\delta$ -receptor gene cloned to date. However, two, overlapping subdivisions of  $\delta$ -receptor have been proposed ( $\delta_1/\delta_2$  and  $\delta_{cx}/\delta_{ncx}$ ) on the basis of *in vivo* and *in vitro* pharmacological experiments. The subdivision of the  $\delta$ -receptor into  $\delta_1$  and  $\delta_2$  subtypes was proposed primarily on the basis of *in vivo* pharmacological studies.

25 The  $\delta_{cx}$  and  $\delta_{ncx}$  subdivision of  $\delta$ -receptors was based on the hypothesis that one type of  $\delta$ -receptor ( $\delta_{cx}$ ) was complexed with  $\mu$ -receptors (and perhaps also  $\kappa$ -receptors) whereas no association with an opioid receptor complex has been observed for the other type of  $\delta$ -receptor ( $\delta_{ncx}$ ) (Rothman *et al.*, *Handbook Exp. Pharmacol.*, A. Herz ed., 104/1:217, 1993).

30 Data obtained from subsequent radioligand binding studies have been

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interpreted as demonstrating the existence of further subtypes of the  $\delta_{ncx}$  receptor, *i.e.*,  $\delta_{(ncx-1)}$  and  $\delta_{(ncx-2)}$ . More recently, it has been suggested that the  $\delta_{(ncx-1)}$  receptor is in fact identical to the  $\delta_1$ -receptor and the  $\delta_{cx}$ -receptor is identical to the  $\delta_2$ -receptor of the previous classification (Xu *et al.*, Peptides, 14:893, 1993).

5

### $\kappa$ -Receptor Subtypes

The situation regarding the proposals for subtypes of the  $\kappa$ -receptor is rather more complex than for the  $\mu$ - and  $\delta$ -receptors, perhaps because of the continuing use of non-selective ligands to define the putative sites. The evidence for the need for sub-division of the  $\kappa$ -receptor comes almost entirely from radioligand binding assays.

10

Studies of  $^3\text{H}$ -ethylketocyclazocine  $^3\text{H}$ -EKC binding in guinea-pig spinal cord pointed to the existence of a non-homogeneous population of high-affinity binding sites, and led to the first proposal for  $\kappa_1$ - and  $\kappa_2$ -sites distinguished by their sensitivity to DADLE (Attali *et al.*, Neuropeptides, 3:53, 1982).

15

Subdivision of the  $\kappa_1$ -site in guinea-pig brain into  $\kappa_{1a}$  and  $\kappa_{1b}$ , was proposed to resolve the complex displacement of either  $^3\text{H}$ -EKC or  $^3\text{H}$ -U-69,593, with dynorphin B and  $\alpha$ -neo-endorphin which both preferentially bound to the proposed  $\kappa_{1b}$  sub-subtype (Clark *et al.*, J. Pharmacol. Exp. Ther., 251:461, 1989). The same study proposed the existence of  $\kappa_3$  subtype, insensitive to U-50,488, that was identified from the binding of  $^3\text{H}$ -naloxone benzoylhydrazone. The pharmacology of this later "  $\kappa_3$ -site " is rather different from the  $\kappa_3$ /MRF site of bovine adrenal medulla, and has been proposed to be the receptor mediating the antinociceptive effect of nalorphine, termed Martin's "N"-receptor (Paul *et al.*, J. Pharmacol. Exp. Ther., 357:1, 1991).

20

Definitive functional pharmacological evidence supporting the existence of this confusing number of putative subtypes of the  $\kappa$ -receptor is lacking, because of the absence of subtype-specific antagonists.

25

\* \* \*

All of this uncertainty and confusion about the precise identity of opioid receptors, and the number of different receptors, has hampered efforts to identify more effective, more specific opioid agonists and antagonists, *i.e.*, more

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specific drugs with fewer untoward side effects, within a large family of neuropharmaceuticals including narcotic analgesics.

Thus, there is a need in the art to identify the molecular basis for the diversity of opioid receptor specificities.

5                   There is a further need to identify specific opioid receptors for screening and development of more effective, less addictive, narcotics.

The present invention addresses these and other needs in the art.

### **SUMMARY OF THE INVENTION**

10                   The present invention relates to the discovery that opioid receptors form functional heterodimers. These receptors can be exploited for high-throughput screening of compounds to identify heterodimer opioid receptor modulators (agonists and antagonists). The invention also relates to identification of novel heterodimer receptor ligands and synergistic compositions, which can form the basis for  
15                   therapeutic strategies to induce analgesia, to combat narcotic addiction, and to reduce hypertension, to name but a few such indications. Moreover, the discovery of certain of these receptor heterodimers has implications for improving immune system function.

                  The present invention provides an isolated heterodimeric receptor,  
20                   which receptor comprises an opioid receptor subunit and a second G-protein coupled receptor (GPCR) subunit. Both receptor subunits are expressed endogenously in the same type of cell. Examples of such heterodimeric receptors include opioid-opioid receptor heterodimers, opioid-dopamine receptor heterodimers, and opioid-adrenergic receptor heterodimers. In specific embodiments, one or both of the receptors is a  
25                   fusion protein comprising an epitope tag (*i.e.*, a peptide segment that is recognized by an antibody).

                  The invention further provides a recombinant host cell that expresses a functional heterodimeric receptor, which receptor comprises an opioid receptor subunit expressed from an expression vector introduced into the host cell, and a  
30                   second G-protein coupled receptor (GPCR) subunit expressed from an expression

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vector introduced into the host cell. Preferably, the host cell stably expresses both receptor subunits.

Also provided is a method of screening for a compound that modulates a property of a heterodimeric receptor as described above. This method comprises  
5 observing a change in a property of the heterodimeric receptor contacted with a candidate compound. For example, receptor trafficking, *e.g.*, internalization, ligand binding, or ligand specificity can be altered in the presence of a test compound. In particular, heterodimeric opioid receptors will exhibit different affinity for various known and test ligands. In particular, the invention provides for testing the activity of  
10 bispecific, bivalent compounds discussed below, or synergistic compositions discussed below.

The invention also advantageously provides a bispecific, bivalent compound comprising an opioid receptor ligand bound to a second G-protein coupled receptor ligand, wherein the second receptor is expressed endogenously in a type of  
15 cell that also endogenously expresses the opioid receptor. Both ligands can be either agonists or antagonists of the specific receptor subunits that make up the heterodimer. Alternatively, antagonist/agonist combinations can provide for synergistic binding as well. For example, this is observed with kappa and delta heterodimeric receptor. In a specific embodiment, based on the surprising discovery made concerning covalent  
20 linkage of kappa receptor homodimers, both ligands of the compound are kappa receptor ligands.

In another aspect, the invention provides a pharmaceutical composition comprising synergistically effective amounts of a ligand of a delta opioid receptor and a ligand of a second receptor selected from the group consisting of kappa opioid  
25 receptor, mu opioid receptor, D2 dopamine receptor, and  $\beta_2$ -adrenergic receptor, and methods for identifying such compositions.

A pharmaceutical composition comprising synergistically effective amounts of a ligand of a kappa opioid receptor and a ligand of a second receptor selected from the group consisting of delta opioid receptor, D2 dopamine receptor,  $\beta_2$ -  
30 adrenergic receptor,  $\alpha_2$ -adrenergic receptor, CCR5, and CXCR4.

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A pharmaceutical composition comprising synergistically effective amounts of a ligand of a mu opioid receptor and a ligand of a second receptor selected from the group consisting of delta opioid receptor, and  $\alpha_2$ -adrenergic receptor.

The compounds and compositions of the invention have heterodimeric opioid receptor modulating properties and thus therapeutic potential. These compounds address the need that exists for more precise identification of receptors for treating a disease or disorder of the central nervous system, cardiovascular system, or immune system. In particular, the disease or disorder may be chronic pain, drug abuse, schizophrenia, depression, or a dysfunction of the central reward pathway. The discovery of a kappa-CCR5 heterodimer provides an avenue for inhibiting HIV infection. The presence of heterodimeric receptors comprising an opioid receptor subunit and a catecholamine receptor subunit identifies a strategy for developing more effective treatments of cardiovascular disease, and in particular hypertension. Administering a therapeutically effective dose of a compound or a pharmaceutical composition of the invention is expected to have a therapeutic effect on such diseases and disorders.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Figures 1A, B, C, D, and E.** Characteristics of kappa opioid receptor homodimers. Immunoblotting of lysates from cells expressing FLAG-kappa receptors or FLAG-delta receptors (**A**). Myc-tagged kappa receptors can be co-precipitated only from cells expressing both myc and FLAG-tagged receptors (**B**) under a variety of extraction conditions and not from a mixture of cells individually expressing these receptors (**C**). Expression of myc- or FLAG-tagged receptors was confirmed by immunoblotting (right panel). Treatment of cells expressing kappa receptors with 1 mM DTT for 30 min followed by 5 mM IAM or NEM results in monomerization (**D**) whereas treatment with 100 nM agonists for 60 min does not (**E**).

**Figures 2A, B, C, and D.** Characterization of kappa-delta heterodimers. Kappa-delta heterodimers can be immunoprecipitated only from myc-kappa and FLAG-delta expressing cells and not from myc-kappa and FLAG-mu

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expressing cells (A). Kappa-delta heterodimers can be immunoprecipitated under a variety of extraction conditions and not from mixture of cells individually expressing these receptors (B). Expression of myc- or FLAG-tagged receptors in each cell line was confirmed by immunoblotting (right panel). Treatment with 5%  $\beta$ -ME for 5 min results in the destabilization of dimers (C). Internalization of receptors in response to 1  $\mu$ M etorphine for 60 minutes (D). Significant differences from untreated controls are indicated, \* $p$ <0.05; \*\*\* $p$ <0.005 (n=3).

**Figures 3A, B, C, D, E, and F.** Ligand binding and functional properties. Competition of  $^3$ H-diprenorphine binding by U-69593 (square), nor-BNI (triangle), diprenorphine (star), DPDPE (circle) and TIPPY (diamond) in membranes from cells expressing kappa (A) delta (B) or kappa and delta (C) receptors. Displacement of  $^3$ H-diprenorphine by U-69593 in the presence of 10  $\mu$ M DPDPE (triangle) or DPDPE in the presence of 10  $\mu$ M U-69593 (diamond) (D). Decrease in intracellular cAMP (E) or increase in phospho-MAP kinase (F) by U-69593 (square), DPDPE (circle) or U-69593+DPDPE (triangle). Activation of homodimers in these cells could account for the effect seen with individual agonists. Error bars represent SEM (n=3-4).

**Figure 4.** Mu and delta receptors interact with each other to form heterodimers. Immunoprecipitation of cell lysates from HEK-293 cells individually expressing either FLAG-mu or myc-delta receptors, mixed cells individually expressing FLAG-mu or myc-delta receptors, or cells co-expressing FLAG-mu and myc-delta was carried out using anti-myc antibodies. Western blotting of these immunocomplexes using anti-FLAG antibodies shows an approximately 150 kDa protein representing mu-delta heterodimers only in cells co-expressing both FLAG-mu and myc-delta receptors. Pretreatment of cells with 1 mM DTT results in the destabilization of dimers.

**Figure 5.** Ligand binding properties in membranes of HEK-cells expressing mu-delta heterodimers were evaluated by measuring the displacement of  $^3$ H-diprenorphine binding by CTOP in cells expressing mu-delta heterodimers. Membranes were incubated with  $^3$ H-diprenorphine in the absence or in the presence

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of various doses of CTOP alone or CTOP in the presence of 1 nM TIPP $\Psi$  as described above. We find a single binding site for CTOP in the absence of TIPP $\Psi$  and a second super high affinity binding site in the presence of 1 nM TIPP $\Psi$ . Data from a representative of experiment (out of 3-7) is shown; error bars represent SEM; variation between experiments was less than 10%.

**Figures 6A, B, C, and D.** Ligand binding properties of whole cells co-expressing mu and delta receptors. Binding of a radiolabeled mu-agonist,  $^3\text{H}$ -DAMGO, examined in the absence or presence of a delta-antagonist, 10 nM TIPP $\Psi$ , in cells exogenously expressing mu and delta receptors (A) or in SK-N-SH neuroblastoma cells that endogenously express mu and delta receptors (B). In the presence of 10 nM TIPP $\Psi$ , a significant (about 50%) increase in the number of  $^3\text{H}$ -DAMGO binding sites is observed. Binding of a radiolabeled mu-agonist,  $^3\text{H}$ -DAMGO, examined in the absence or presence of two subtype delta selective agonists, deltorphin II (C) and DPDPE (D). The increase in the population of receptors is seen only in the presence of deltorphin II and not in the presence of DPDPE. Data in panels A-D represent mean $\pm$ SEM from 3-5 independent experiments.

**Figures 7A, B, and C.** Delta opioid receptors interact with  $\beta_2$ -adrenergic receptors (A), with D2 dopamine receptors (B), or with  $\alpha_2\text{A}$ -adrenergic receptors (C) to form heterodimers. A. Western blotting (left and right panels) and immunoprecipitation (middle panel) of cell lysates from mixed HEK-293 cells individually expressing either FLAG- $\beta_2$ -adrenergic receptors or *myc*-delta receptors, or cells co-expressing FLAG- $\beta_2$ -adrenergic receptors and *myc*-delta receptors was carried out using either anti-FLAG or anti-*myc* antibodies. Western blotting of anti-*myc*-precipitated immunocomplexes with anti-FLAG antibodies shows delta- $\beta_2$  heterodimers only in cells co-expressing both FLAG- $\beta_2$  and *myc*-delta receptors. B. Western blotting (left and right panels) and immunoprecipitation (middle panel) of cell lysates from mixed HEK-293 cells individually expressing either FLAG-D2 dopamine receptors or *myc*-delta receptors, or cells co-expressing FLAG-D2

-10-

dopamine receptors and *myc*-delta receptors was carried out using either anti-FLAG or anti-*myc* antibodies. Western blotting of anti-*myc*-precipitated immunocomplexes with anti-FLAG antibodies shows delta-D2 heterodimers only in cells co-expressing both FLAG-D2 and *myc*-delta receptors. C. Western blotting following the

5 immunoprecipitation of cell lysates from mixed HEK-293 cells individually expressing either HA- $\alpha_2$ A-adrenergic receptors or *myc*-delta receptors (left panel), or from cells co-expressing HA- $\alpha_2$ A-adrenergic receptors and *myc*-delta receptors (right panel). Western blotting of anti-HA-precipitated immunocomplexes was performed using anti-*myc* antibodies and shows delta- $\alpha_2$ A heterodimers only in cells co-

10 expressing both HA- $\alpha_2$ A-adrenergic receptors and *myc*-delta receptors.

#### **DETAILED DESCRIPTION OF THE INVENTION**

For the first time, a heterodimeric receptor having at least one opioid receptor moiety or subunit has been characterized. The invention is based, in part, on

15 biochemical and pharmacological evidence for the heterodimerization of fully functional opioid receptors (kappa and delta; and mu and delta), and fully functional opioid receptors/catecholamine receptors (delta and  $\beta_2$ -adrenergic receptor [ $\beta_2$ -AR]; kappa and D2; and delta and D2). Discovery of these receptors opens the door to the preparation of bispecific compounds and synergistic compositions of individual

20 receptor ligands, based on the discovery of modified ligand specificity of these heterodimeric receptors and synergistic binding of receptor subunit-specific ligands. The present invention has uncovered the mechanism for synergy of different classes of drugs, which, though observed empirically, has not been satisfactorily explained until now. Thus, the present invention advantageously provides for the development

25 both of more potent therapeutic agents and of a better understanding of the molecular basis of opioid receptor activity.

As used herein, the term "receptor subunit" refers to a single receptor protein that has been discovered to associate with another, different G-protein coupled receptor (GPCR), which is either a different opioid receptor protein or a non-opioid

30 receptor, such as dopamine receptor, adrenergic receptor, or chemokine receptor. For

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example, in a kappa-delta opioid heterodimeric receptor, the kappa opioid receptor protein and the delta opioid receptor protein that form the heterodimer are each subunits.

The term "opioid receptor" refers to a "classical" type of opioid  
5 receptor: mu ( $\mu$ ), delta ( $\delta$ ), and kappa ( $\kappa$ ). A "heterodimeric opioid receptor" or "opioid receptor heterodimer" refers to a G-protein coupled receptor of the invention, comprising an opioid receptor subunit and another GPCR receptor subunit, which maybe another opioid receptor subunit or a non-opioid receptor subunit.

Receptors are "expressed endogenously" in a type of cell that  
10 endogenously does or does not express the opioid receptor or are both expressed endogenously in the same type of cell (*i.e.*, expressed together with a different opioid receptor(s) or a different GPCR [*e.g.*, catecholamine receptor]). While such co-expression may have been recognized in the scientific literature, the discovery of the present invention represents the first indication that the receptor subunits form a  
15 functional heterodimer.

Non-limiting examples of endogenous co-expression of opioid  
receptors with other GPCRs include kappa and the D2 dopamine receptor; delta and D2; mu and delta; mu and  $\alpha_2$ -adrenergic receptor; delta and  $\alpha_2$ -adrenergic receptor;  
20 delta and  $\beta_2$ -adrenergic receptor; kappa and delta; kappa and  $\beta_2$ -adrenergic receptor; kappa and ORL<sub>1</sub> (nociceptin) receptor; delta and ORL<sub>1</sub> (nociceptin) receptor; and kappa and the CCR5 and CXCR4 chemokine receptors.

The term "functional" refers to the ability to accomplish at least one of the following: bind ligand; bind a "non-selective" ligand selectively; bind a bifunctional, bi-specific ligand; activate G-protein mediated signal transduction upon  
25 binding a ligand; induce internalization of the receptor; or any combination thereof. Such ability or abilities can be demonstrated in a cell based or cell-free system, or *in vivo*, including a transgenic animal system.

A bi-specific, bivalent compound of the invention comprises ligands for each of the individual heterodimeric receptor subunits bound to each other.

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A "synergistically effective amount" of a ligand is an amount able to induce heterodimeric opioid receptor activity at a much lower concentration, particularly a subthreshold concentration, than is required for homodimeric (classical) receptor activity by virtue of the presence of a ligand of a different receptor, which is a subunit of the heterodimeric receptor. So, if ligand A is used, ligand B can be effective at a lower concentration than in the absence of A, and similarly when B is used, A can be effective at a lower concentration than in the absence of B. Although not intending to be bound by any particular explanation or theory, binding of one ligand to the heterodimeric receptor may alter conformation or otherwise facilitate recognition and binding of the other ligand. Thus, both ligands become surprisingly and unexpectedly effective at concentrations generally regarded as subthreshold (or subtherapeutic), even though the ligands themselves do not induce the same effects. Thus, any increase in efficacy represents a synergy, since there is no basis for expecting additive effects, *e.g.*, with an opioid and a catecholamine.

Furthermore, the term "synergistic" can be used to describe an activity that two ligands have together that neither one has alone or in the absence of the other.

The term "signal transduction pathway" as used in this invention refers to the intracellular mechanism by which an opioid or other heterodimeric receptor ligand induces an alteration of cell function or activity. A key feature of the signal transduction pathway dissected herein is activation of G-protein coupled signaling, such as cAMP production, resulting in further signal transduction, including MAPK phosphorylation.

The term "element of a signal transduction pathway" refers to a signal transduction factor that is activated as a result of ligand binding to a heterodimeric receptor. In accordance with the present invention, elements of the signal transduction pathway include G-proteins, cAMP, MAPK, etc. A "signal" in such a pathway can refer to activation of an element (or factor) in the pathway. For example, activation of MAPK is a signal of agonist-induced kappa-delta heterodimer signal transduction pathway. Generally, activation of one of these factors involves phosphorylation of one or more proteins.

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The term "inhibitor" is used herein to refer to a compound that can block or reduce the level of signaling in a signal transduction pathway described herein. Such an inhibitor may block the pathway at any point, from blocking binding of ligand to receptor to blocking function of intracellular signals. Preferably, an inhibitor discovered in accordance with the invention is specific for signals of heterodimeric opioid receptor-induced signalling.

"Screening" refers to a process of testing one or a plurality of compounds (including a library of compounds) for some activity. A "screen" is a test system for screening. Screens can be primary, *i.e.*, an initial selection process, or secondary, *e.g.*, to confirm that a compound selected in a primary screen (such as a binding assay) functions as desired (such as in a signal transduction assay). Screening permits the more rapid elimination of irrelevant or non-functional compounds, and thus selection of more relevant compounds for further testing and development. "High throughput screening" involves the automation and robotization of screening systems to rapidly screen a large number of compounds for a desired activity. Screens are discussed in greater detail below.

As used herein, the term "isolated" means that the referenced material is removed from its native environment, *e.g.*, a cell. Thus, an isolated biological material can be free of some or all cellular components, *i.e.*, components of the cells in which the native material occurs naturally (*e.g.*, cytoplasmic or membrane component). A material shall be deemed isolated if it is present in a cell extract or if it is present in a heterologous cell or cell extract. In the case of nucleic acid molecules, an isolated nucleic acid fragment includes a PCR product, an isolated mRNA, a cDNA, or a restriction fragment. In another embodiment, an isolated nucleic acid fragment is preferably excised from the chromosome in which it may be found, and more preferably is no longer joined or proximal to non-coding regions (but may be joined to its native regulatory regions or portions thereof), or to other genes, located upstream or downstream of the gene contained by the isolated nucleic acid molecule when found in the chromosome. In yet another embodiment, the isolated nucleic acid fragment lacks one or more introns. Isolated nucleic acid molecules

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include sequences inserted into plasmids, cosmids, artificial chromosomes, and the like, *i.e.*, when it forms part of a chimeric recombinant nucleic acid construct. Thus, in a specific embodiment, a recombinant nucleic acid is an isolated nucleic acid fragment. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it is a membrane-associated protein. An isolated organelle, cell, or tissue is removed from the anatomical site in which it is found in an organism. An isolated material may be, but need not be, purified.

The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate the presence of unrelated materials, *i.e.*, contaminants, including native materials from which the material is obtained. For example, a purified protein is preferably substantially free of other proteins or nucleic acids with which it is associated in a cell; a purified nucleic acid molecule is preferably substantially free of proteins or other unrelated nucleic acid molecules with which it can be found within a cell. As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 50% pure; more preferably, at least 90% pure, and more preferably still at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, and other methods known in the art.

Methods for purification are well-known in the art. For example, nucleic acids can be purified by precipitation, chromatography (including without limitation preparative solid phase chromatography, oligonucleotide hybridization, and triple helix chromatography), ultracentrifugation, and other means. Polypeptides and proteins can be purified by various methods including, without limitation, preparative disc-gel electrophoresis and isoelectric focusing; affinity, HPLC, reversed-phase HPLC, gel filtration or size exclusion, ion exchange and partition chromatography; precipitation and salting-out chromatography; extraction; and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that

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facilitates purification, such as, but not limited to, a polyhistidine sequence, or a sequence that specifically binds to an antibody, such as FLAG, *myc*, HA, and GST. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies  
5 produced against the protein or against peptides derived therefrom can be used as purification reagents. Cells can be purified by various techniques, including centrifugation, matrix separation (*e.g.*, nylon wool separation), panning and other immunoselection techniques, depletion (*e.g.*, complement depletion of contaminating cells), and cell sorting (*e.g.*, fluorescence activated cell sorting (FACS)). Other  
10 purification methods are possible and contemplated herein. A purified material may contain less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components, media, proteins, or other nondesirable components or impurities (as context requires), with which it was originally associated. The term "substantially pure" indicates the highest degree of purity which  
15 can be achieved using conventional purification techniques known in the art.

In a specific embodiment, the term "about" or "approximately" means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. Alternatively, logarithmic terms used in biology, the term "about" can mean within an order of magnitude of a given value, and preferably within one-half an order  
20 of magnitude of the value.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*,  
25 Second Edition, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II, D.N. Glover ed., 1985; *Oligonucleotide Synthesis*, M.J. Gait ed., 1984; *Nucleic Acid Hybridization*, B.D. Hames and S.J. Higgins eds., 1985; *Transcription And Translation*, B.D. Hames and S.J. Higgins, eds., 1984; *Animal Cell*  
30 *Culture*, R.I. Freshney ed., 1986; *Immobilized Cells And Enzymes*, IRL Press, 1986;

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B. Perbal, *A Practical Guide To Molecular Cloning*, 1984; *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.* eds., John Wiley and Sons, Inc., 1994.

**Molecular Biology - Definitions**

5                   A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

10                   The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays, as described *infra*.

15                   A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.*, the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

20                   The coding sequences for individual receptors may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, intros, 5'- and 3'- non-coding regions, and the like.

25                   The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins, and may or may not include regulatory

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DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" or "operatively associated with" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, *e.g.*, the resulting protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

The term "transfection" means the introduction of a foreign nucleic acid into a cell. The term "transformation" means the introduction of a "foreign" (*i.e.*, extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance,

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typically a protein or enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (*e.g.*, a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.*, transcription and translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc.; they are discussed in greater detail below.

Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that

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encodes a particular amino acid sequence for a particular protein or enzyme.

Promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET plasmids (Invitrogen, San Diego, CA), pCDNA3 plasmids (Invitrogen), pREP plasmids (Invitrogen), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.*, antibiotic resistance, and one or more expression cassettes.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.*, for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Expression systems include mammalian host cells and vectors. Suitable cells include PC12 cells, CHO cells, HeLa cells, 293 and 293T (human kidney cells), COS cells, mouse primary myoblasts, and NIH 3T3 cells.

The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is a such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, the opioid receptor gene and a second receptor gene are heterologous to the vector or vectors in which they are inserted for cloning or expression, and they are heterologous to a host cell containing such a vector, in which it is expressed, *e.g.*, a CHO cell.

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The terms "mutant" and "mutation" mean any detectable change in genetic material, *e.g.*, DNA, or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (*e.g.*, DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.*, protein or enzyme) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.*, any kind of mutant.

"Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Such variants can be used in expression of receptor subunits, *e.g.*, where altered codon usage or insertion of a restriction site is desired.

"Function-conservative variants" are those in which a given amino acid residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Such changes are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. Amino acids other than those indicated as conserved may differ in a protein or enzyme so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, most preferably at least 85%, and even more preferably at

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least 90%, and which has the same or substantially similar properties or functions as the native or parent protein or enzyme to which it is compared. Any of these algorithms can be used with defaults provided by the manufacturer, supplier, or provider.

5                    Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acids are identical, or greater than about 90% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual  
10    for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program, or any of the programs described above (BLAST, FASTA, etc.)

                  A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the  
15    appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, 1989, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a  $T_m$  (melting temperature) of 55°C, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no  
20    formamide; or 30% formamide, 5x SSC, 0.5% SDS). Equivalent stringency conditions can be achieved by increasing melting temperature and lowering salt concentration, or *vica versa*. Moderate stringency hybridization conditions correspond to a higher  $T_m$ , *e.g.*, 40% formamide, with 5x or 6x SSC. High stringency hybridization conditions correspond to the highest  $T_m$ , *e.g.*, 50% formamide, 5x or 6x  
25    SSC. SSC is a 0.15M NaCl, 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art.  
30    The greater the degree of similarity or homology between two nucleotide sequences,

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the greater the value of  $T_m$  for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher  $T_m$ ) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating  $T_m$  have been derived (see  
5 Sambrook *et al.*, 1989, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook *et al.*, 1989, *supra*, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the  
10 length is at least about 20 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a  $T_m$  of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the  $T_m$  is 60°C; in a more preferred embodiment, the  $T_m$  is 65°C. In a specific embodiment, "high stringency" refers to hybridization and/or washing  
15 conditions at 68°C in 0.2XSSC, at 42°C in 50% formamide, 4XSSC, or under conditions that afford levels of hybridization equivalent to those observed under either of these two conditions.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20  
20 nucleotides, preferably no more than 100 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, with  $^{32}\text{P}$ -nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a  
25 probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, *e.g.*, for cloning full length or a fragment of a receptor. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog  
30 bonds, such as thioester bonds, etc.

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Vectors

A wide variety of host/expression vector combinations may be employed in expressing DNA sequences encoding the heterodimeric opioid receptor subunits. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include SV40 and derivatives of SV40 and bacterial plasmids, *e.g.*, *E. coli* plasmids col E1, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, Gene, 67:31-40, 1988), pMB9 and their derivatives, plasmids such as RP4; phage DNAs, *e.g.*, the numerous derivatives of phage  $\lambda$ , *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 $\mu$  plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

A vector can be introduced *in vivo* in a non-viral vector, *e.g.*, by lipofection, with other transfection facilitating agents (peptides, polymers, etc.), or as naked DNA. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection, with targeting in some instances (Felgner, *et al.*, Proc. Natl. Acad. Sci. USA, 84:7413-7417, 1987; Felgner and Ringold, Science, 337:387-388, 1989; Mackey, *et al.*, Proc. Natl. Acad. Sci. USA, 85:8027-8031, 1988; Ulmer *et al.*, Science, 259:1745-1748, 1993). Useful lipid compounds and compositions for transfer of nucleic acids are described in PCT Publication Nos. WO 95/18863 and WO 96/17823, and in U.S. Patent No. 5,459,127. Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, PCT Publication No. WO 95/21931), peptides derived from DNA binding proteins (*e.g.*, PCT Publication No. WO 96/25508), or a cationic polymer (*e.g.*, PCT Publication No. WO 95/21931). Recently, a relatively low voltage, high efficiency *in vivo* DNA transfer technique, termed electrotransfer, has been described (Mir *et al.*, C.P. Acad. Sci., 321:893, 1998; PCT Publications Nos. WO 99/01157; WO 99/01158; WO 99/01175). DNA vectors for gene therapy can be introduced into the desired host

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cells by methods known in the art, *e.g.*, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun (ballistic transfection), or use of a DNA vector transporter (*see, e.g.*, Wu *et al.*, J. Biol. Chem., 267:963-967, 1992; Wu and Wu, J. Biol. Chem., 263:14621-14624, 1988; Canadian Patent Application No. 2,012,311; Williams *et al.*, Proc. Natl. Acad. Sci. USA, 88:2726-2730, 1991). Receptor-mediated DNA delivery approaches can also be used (Curiel *et al.*, Hum. Gene Ther. 3:147-154, 1992; Wu and Wu, J. Biol. Chem. 262:4429-4432, 1987). US Patent Nos. 5,580,859 and 5,589,466 disclose delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal.

Also useful are viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia virus, baculovirus, and other recombinant viruses with desirable cellular tropism. Thus, a gene encoding a functional protein or polypeptide (as set forth above) can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in PCT Publication No. WO 95/28494.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (*see, e.g.*, Miller and Rosman, BioTechniques, 7:980-990, 1992). Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques

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may be performed *in vitro* (on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably, the replication defective virus retains the sequences of its genome which are necessary for encapsidating the viral particles.

5 DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for  
10 administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt *et al.*, Molec. Cell. Neurosci., 2:320-330, 1991), defective herpes virus vector lacking a glyco-protein L gene (Patent Publication RD 371005 A), or other  
15 defective herpes virus vectors (PCT Publication Nos. WO 94/21807 and WO 92/05263); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* (J. Clin. Invest., 90:626-630, 1992; *see also* La Salle *et al.*, Science, 259:988-990, 1993); and a defective adeno-associated virus vector (Samulski *et al.*, J. Virol., 61:3096-3101, 1987; Samulski *et al.*, J. Virol., 63:3822-3828, 1989;  
20 Lebkowski *et al.*, Mol. Cell. Biol., 8:3988-3996, 1988).

Various companies produce viral vectors commercially, including but by no means limited to Avigen, Inc. (Alameda, CA; AAV vectors), Cell Genesys (Foster City, CA; retroviral, adenoviral, AAV vectors, and lentiviral vectors), Clontech (retroviral and baculoviral vectors), Genovo, Inc. (Sharon Hill, PA;  
25 adenoviral and AAV vectors), Genvec (adenoviral vectors), IntroGene (Leiden, Netherlands; adenoviral vectors), Molecular Medicine (retroviral, adenoviral, AAV, and herpes viral vectors), Norgen (adenoviral vectors), Oxford BioMedica (Oxford, United Kingdom; lentiviral vectors), and Transgene (Strasbourg, France; adenoviral, vaccinia, retroviral, and lentiviral vectors).

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A preferred expression host is a eukaryotic cell (*e.g.*, yeast, insect, or mammalian cell). More preferred is a mammalian cell, *e.g.*, human, rat, monkey, dog, or hamster cell. In specific embodiments, *infra*, opioid receptor heterodimers are expressed in a human embryonic kidney (HEK) 293 line or a COS line. Other choices  
5 include a cell line (*e.g.*, SK-N-MC), or a chinese hamster ovary cell line (*e.g.*, CHO-K1).

Expression of one or both receptor subunits may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used for gene  
10 expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist and Chambon, 1981, *Nature*, 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell*, 22:787-797, 1980), the herpes thymidine kinase promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:1441-1445,  
15 1981), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature*, 296:39-42, 1982); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Kamaroff *et al.*, *Proc. Natl. Acad. Sci. USA*, 75:3727-3731, 1978), or the *tac* promoter (DeBoer *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:21-25, 1983); *see also* "Useful proteins from recombinant bacteria" in *Scientific American*, 242:74-94, 1980;  
20 promoter elements from yeast or other fungi such as the Gal4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, *Cell*, 38:639-646, 1984;  
25 Ornitz *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 50:399-409, 1986; MacDonald, *Hepatology*, 7:425-515, 1987); insulin gene control region which is active in pancreatic beta cells (Hanahan, *Nature*, 315:115-122, 1985), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, *Cell*, 38:647-658, 1984; Adames *et al.*, *Nature*, 318:533-538, 1985; Alexander *et al.*, *Mol. Cell. Biol.*,  
30 7:1436-1444, 1987), mouse mammary tumor virus control region which is active in

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testicular, breast, lymphoid and mast cells (Leder *et al.*, Cell, 45:485-495, 1986), albumin gene control region which is active in liver (Pinkert *et al.*, Genes and Devel., 1:268-276, 1987), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, Mol. Cell. Biol., 5:1639-1648, 1985; Hammer *et al.*, Science, 5 235:53-58, 1987), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, Genes and Devel., 1:161-171, 1987), beta-globin gene control region which is active in myeloid cells (Mogam *et al.*, Nature, 315:338-340, 1985; Kollias *et al.*, Cell, 46:89-94, 1986), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, Cell, 48:703-712, 1987), myosin 10 light chain-2 gene control region which is active in skeletal muscle (Sani, Nature, 314:283-286, 1985), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, Science, 234:1372-1378, 1986).

### **Specific Receptor Combinations**

15 Various heterodimeric receptor combinations, based on endogenous co-expression of the receptor subunits, are specifically contemplated by the invention. Specific examples are set forth below. In these instances, the existence of a heterodimeric receptor form accounts for the observed characteristics of ligand binding. Thus, in the section that follows, the boldface headings are contributed by 20 the present invention. The explanatory text explains the basis for identification of the heterodimeric receptor of the invention based on evidence for co-expression of each receptor subunit in the cell, and in some instances by previously unexplained pharmacological data.

The identification of these specific receptor combinations provides a 25 basis for identifying heterodimeric receptor ligands that modulate cellular activity. Modulation of the heterodimeric receptor combinations with such ligands will provide a strategy for affecting various neurological and physiological changes *in vivo*.

It bears noting that although the following receptors have been identified as co-expressed in cells, and in some cases there is evidence for co-

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modulation of ligand binding, there is no indication of the possible existence of a heterodimeric opioid receptor of the invention.

**Kappa and D2:** The noradrenergic neurons of the autonomic nervous and the central nervous system are endowed with presynaptic receptors by which  
5 noradrenaline release is inhibited by noradrenaline itself (via the  $\alpha_2$ -autoreceptor) and by other transmitters and mediators (via heteroreceptors). Frequently, inhibitory interactions exist between auto- and heteroreceptors, as is the case for the following heteroreceptors: adenosine A1, cannabinoid CB1, dopamine D2/D3, histamine H3, 5-hydroxytryptamine (serotonin) 5-HT (1B), imidazoline muscarine M2, delta opioid,  
10 kappa opioid, mu opioid, orphan opioid (ORL1), prostaglandin EP3 and somatostatin SRIF1. Such interactions may prevent the identification of a putative heteroreceptor or the quantitative estimation of the effect mediated by this receptor (Schlicker and Gothert, Brain Res. Bull., 47:129, 1998).

Studies on rats, where U-69593, the selective  $\kappa$ -opioid agonist, was  
15 repeatedly administered in daily injections, demonstrated that kappa-opioid agonists induce long-term alterations in dopamine D2 receptors. Furthermore, the finding that these changes in receptor number require repeated injections and a withdrawal time of one day suggests that these alterations are compensatory in nature (Izenwasser *et al.*, Synapse, 30:275, 1998).

20 The presynaptic regulation of stimulated dopamine release from superfused rat striatal synaptosomes by opioids and GABA has been studied. It was found that dopamine D2 autoreceptors were inhibited through activation of a homogenous population of kappa-opioid receptors in view of the potent inhibitory effect of kappa-selective agonist U-69593 (Ronken *et al.*, J Neurochem., 61:1634,  
25 1993).

The present invention provides the first biochemical and pharmacological evidence for the heterodimerization of the fully functional kappa opioid receptor and the dopamine D2 receptor. As described in more detail in the Example 3 (*infra*), this heterodimerization was demonstrated using co-expression of  
30 the tagged versions of the two receptors in cultured cells (that normally don't express

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these receptors), followed by immunoprecipitation of receptor heterodimers with anti-tag antibodies. Kappa-dopamine D2 heterodimers were observed only in lysate from cells co-expressing the two receptors together, and not in the lysate from a mixture of cells individually expressing these receptors. From ligand binding studies it was also shown that, when administered together, highly selective ligands of the two receptors (agonists or antagonists) bound co-operatively and synergistically potentiated the hetero-receptor function.

**Kappa and  $\beta_2$ :** The present invention also provides the first biochemical and pharmacological evidence for the heterodimerization of the fully functional kappa opioid receptor and the  $\beta_2$ -adrenergic receptor. The experimental approach was identical to the one employed for kappa-dopamine D2 heterodimers, and is described in more detail in the Example 3 (*infra*).

**Kappa and Delta:** According to the present invention the ability of kappa receptors to heterodimerize with delta receptors was shown by co-expressing *myc*-tagged kappa receptors with FLAG-tagged delta receptors in cultured cells, followed by immunoprecipitation using antibodies specific for *myc*-tagged kappa receptors (described in more detail in the Example 1, *infra*). It was also shown that kappa-delta heterodimers are destabilized by a reducing agent indicating that disulfide bonds are involved in kappa-delta heterodimerization.

The present invention also addressed the issue of functional significance of kappa-delta heterodimerization. Thus, the pronounced effect of heterodimerization on receptor trafficking (*i.e.*, internalization) was demonstrated using cells co-expressing kappa and delta receptors and etorphine, a potent non-selective opioid agonist that binds both delta and kappa receptors with high affinity. In addition, the results of ligand binding/competition assays imply that kappa-delta heterodimerization produces a new binding site which is able to synergistically bind highly selective ligands. The synergistic activation of heterodimeric receptor by selective ligands is reflected in a significant potentiation of signal transduction pathways (*e.g.*, cAMP level reduction, activation of MAPK), and

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suggests that the kappa-delta heterodimer represents a functional receptor with novel properties.

**Delta and D2:** Co-expression of these receptors was reported in, *inter alia*, Fuxe *et al.*, *The Opioid Peptide Systems: Their Organization and Role in Volume Transmission and Neuroendocrine Regulation*. In: *Regulatory Roles of opioid Peptides*, Illes, P., Farsang, C Weinham eds., New York, pp 33-68, 1988. The present invention provides the first biochemical and pharmacological evidence for the heterodimerization of the fully functional delta opioid receptor and the dopamine D2 receptor. The experimental approach was identical to the one employed for kappa-dopamine D2 heterodimers (*supra*), and is described in more detail in the Example 3 (*infra*).

**Mu and Delta:** Dopamine D1 receptor-stimulated cyclic AMP efflux from superfused neostriatal slices was strongly inhibited by the delta-opioid receptor agonist [D-Pen2, D-Pen5] enkephalin (DPDPE, 1 $\mu$ M) and by the mu-opioid receptor agonist [D-Ala2, MePhe4, Gly-ol5] enkephalin (DAGO, 1 $\mu$ M). The data indicate that FIT (fentanyl isothiocyanate) and Naloxone, acting on delta and mu receptors, respectively, may share a common binding site (Schoffelmeer *et al.*, Eur. J. Pharmacol., 149:179, 1988), suggesting the involvement of a functional mu-delta opioid receptor complex.

Previous studies delineated two classes of delta binding sites; a delta not associated with the opioid receptor complex, termed the delta ncx site, and a delta site associated with the opioid receptor complex, termed the delta cx site. The main findings were that the pretreatment of membranes with (+)-trans-SUPERFIT, a delta selective acylating agent, decreased the IC50 values (*i.e.*, concentration required for 50% inhibition of binding) of delta-preferring drugs, and increased the IC50 values of mu-preferring drugs, for the delta cx binding site. Mu preferring drugs were non-competitive inhibitors of <sup>3</sup>H[D-Ala, Leu5] enkephalin binding to the delta cx site, delta-preferring drugs were competitive inhibitors (Rothman *et al.*, Peptides, 13:1137, 1992).

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Acute antinociceptive effects of opioid mu agonists were shown to be modulated by delta agonists, while the development of antinociceptive tolerance was not (Jiang *et al.*, Eur. J. Pharmacol., 186:137, 1990).

Radioligand binding assays and functional experiments revealed that  
5 the SK-N-BE neuroblastoma cell line expresses a similar ratio of mu and delta opioid receptors, both negatively coupled to adenylyl cyclase through pertussis toxin-sensitive G-proteins (Palazzi *et al.*, J. Neurochem., 67:138, 1996). The findings here indicate that some functional interaction occurred between the two opioid subtypes. In fact, a long term exposure to DAMGO, a mu-selective agonist,  
10 sensitized the functional response of the delta-selective agonist, but not vice versa. These data supports the hypothesis of the existence of cross-talk between mu and delta receptors in the SK-N-BE cell line.

Morphine is known to bind primarily to mu receptors; this is supported by the total lack of response to morphine in transgenic animals lacking mu opioid  
15 receptors (Mathes *et al.*, Nature, 383:819-823, 1996 ). Interestingly, in these animals the delta ligand-mediated analgesia is also altered suggesting an interaction between these two receptors (Sora *et al.*, Eur J. Pharmacol., R1-R29, 1997). Likewise, in wild-type animals chronic morphine treatment was found to selectively upregulate a subpopulation of delta opioid receptors (Rothman *et al.*, Eur. J. Pharm., 160:71-82,  
20 1989; Rothman *et al.*, Eur. J. Pharm., 124:113-119, 1986). When opioid receptor roles in the analgesia were assessed using classical delta-opioid receptor agonist, DPDPE, analgesia was dramatically reduced in mu-opioid receptor knockout mice in a gene-dose dependent fashion (Sora *et al.*, *supra*).

In addition, immunohistochemical studies of the opioid receptor  
25 distribution in the CNS have shown that mu and delta receptors co-localize to the same axonal terminals of the superficial dorsal horn (Mohler and Fritschy, Trends in Pharmacol. Sci., 20:87-89, 1999; Vaught *et al.*, Life Sci., 30:1443-1445, 1982).

As detailed in Example 2 (*infra*), the present invention describes the isolation (by immunoprecipitation) of approximately 150 kDa mu-delta heterodimer  
30 from the cultured cells expressing both receptors in their tagged forms. Examination

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of the ligand binding properties of the mu-delta heterodimer in cells expressing both receptors revealed the synergistic binding of receptor type selective ligands suggesting the formation of a heterodimer-specific novel binding site. Morphine and other opiates were demonstrated to bind this site with super high affinity.

- 5                   **Mu and  $\alpha_2$ :** Mu-opioid receptor efficacy appears to be dependent on the degree of activation of  $\alpha_2$ -adrenoceptors in central noradrenergic nerve terminals, possibly through a local receptor interaction within the nerve terminal membrane (Schoffelmeeer *et al.*, N. S. Arch. Pharmacol., 333:377, 1986).

- 10                   In pontine slices of the rat brain, the frequency of the spontaneous action potentials of locus coeruleus (LC) neurons was recorded extracellularly. The spontaneous activity of LC neurons was inhibited by somatic alpha 2-adrenoceptors and opioid mu-receptors. It has been suggested that the two receptors interact with each other at a site located between themselves and not in the subsequent common signal transduction system (Illes and Norenberg, N. S. Arch. Pharmacol., 342:490, 15                   1990).

- A number of *in vivo* studies have shown that  $\alpha_2$ -adrenergic receptor modulates opioid effects (Aley and Levine, J. Neurosci., 17:735-744, 1997; Bentley *et al.*, Br. J. Pharmacol., 79:125-134, 1983; Bucher *et al.*, N. S. Arch Pharmacol., 345:37-43, 1992; Stone *et al.*, J Neurosci., 17:7157-65, 1997). One of these studies 20                   (Aley and Levine, *supra*) showed that morphine was able to modulate the activity of  $\alpha_2$ -adrenergic receptor and not of the A1 adenosine receptor. This receptor cross talk is hypothesized to be either via second messenger systems or via physical association of the receptors. However, the asymmetric interactions as observed by morphine's ability to modulate the activity of  $\alpha_2C$ -adrenergic receptor and not of the A1 25                   adenosine receptor, although both receptors negatively coupled to adenylate cyclase, has led to the proposal that the receptors are physically associated on the membranes and that the mu receptors have the ability to complex with  $\alpha_2C$ -adrenergic receptor and not with the A1 adenosine receptor (Darland and Grandy, Br. J. Anaesth., 81:29-37, 1998 ). In support of this, studies carried out by Stone *et al.* (*supra*) have shown 30                   that the  $\alpha_2A$ -adrenergic receptor subtype (a point mutation in the  $\alpha_2A$ -adrenergic

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receptor) is the primary mediator of  $\alpha_2$ A-adrenergic spinal analgesia and is necessary for analgesic synergy with opioids. This synergy between opiates and adrenergic drugs in mediating spinal analgesia is lost in transgenic animals lacking functional adrenergic receptor. The present invention provides the first biochemical and pharmacological evidence for the heterodimerization of the fully functional delta opioid receptor and  $\alpha_2$ A-adrenergic receptor. The experimental approach was identical to the one employed in kappa-D<sub>2</sub> heterodimers, and is described in detail in Example 3 (*infra*).

**Kappa and  $\alpha_2$ :** The interaction of presynaptic, release inhibiting alpha 2-adrenoceptors, opioid kappa-receptors and adenosine A1-receptors was studied in slices of the parieto-occipital cortex of rabbits. The selective kappa-receptor agonist ethylketocyclazocine (EK) attenuated markedly the release-inhibiting effects of the  $\alpha_2$ -adrenoceptor-selective agonists and antagonists (Limberger *et al.*, N. S. Arch Pharmacol 338:53, 1988). It was concluded that there is an interaction between presynaptic  $\alpha_2$ -adrenoceptors and opioid kappa-receptors, either at the level of the receptors themselves or of the post-receptor reaction chains.

**Delta and  $\beta_2$ :** Cardiac myocyte sarcolemma contains both catecholamine and opioid peptide receptors (OPRs). Potent inhibitory "cross-talk" between delta-OPR and  $\beta$ -adrenergic receptor signaling pathways was found to occur via a PTX (pertussis toxin) sensitive G-protein involved in adenylyl cyclase inhibition in rat heart (Pepe *et al.*, Circulation, 95:2122, 1997), suggesting the interaction between delta-OPR and  $\beta$ -adrenergic receptor.

The present invention provides the first biochemical and pharmacological evidence for the heterodimerization of the fully functional delta opioid receptor and the  $\beta_2$ -adrenergic receptor. The experimental approach was identical to the one employed for kappa-dopamine D2 heterodimers (*supra*), and is described in more detail in the Example 3 (*infra*).

**Kappa and CCR5 or CXCR4:** Co-expression of these receptor types has been previously reported (Chao *et al.*, Proc. Natl. Acad. Sci. USA, 923:8051-6, 1996). The isolation of opioid receptor dimers with other opioid, dopamine, and adrenergic receptors, which is disclosed in

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the present invention, suggests the possibility of formation of kappa-CCR5 and kappa-CXCR4 heterodimers. Given the approaches disclosed in the present invention, anyone skilled in the art would be in the position to demonstrate the existence of kappa-CCR5 and kappa-CXCR4 heterodimers experimentally. The  
5 existence of such heterodimers will, in turn, explain a previously reported feedback of opiates on immune system function. It will also provide a solid basis for using opioids as competitors for undesirable chemokine receptor interactions. Primary among these undesirable interactions is the interaction of HIV with CCR5 for intracellular transport.

10           **Opioid Receptor and Nociceptin Receptor:** Another candidate for heterodimerization with opioid receptors is a recently identified nociceptin receptor. The cDNA for nociceptin (also designated orphanin FQ) receptor was isolated based on the homology to opioid receptors (Civelli *et al.*, Crit. Rev. Neurobiol., 12,163-76, 1998). A search for ligands that activate this receptor led to the identification of  
15 nociceptin (orphanin FQ) peptides (Darland and Grandy, *supra*). Pharmacological and behavioral studies have shown that these peptides modulate opiate-mediated analgesia (Civelli *et al.*, *supra*).

#### Screening Assays

20           As exemplified in the Examples, *infra*, the present invention provides various screening assays for modulation of heterodimeric opioid receptor activation. The assays of the invention are particularly advantageous by permitting rapid evaluation of cellular responses. Biological assays, which depend on testing perception, pain sensitivity, survival, or some other response *in vivo* require  
25 substantial amounts of time and resources to evaluate. By detecting individual signals in the signal transduction pathway, the present invention short-circuits the more tedious and time consuming biological assays. Furthermore, the signal transduction assays can often be performed with very small amounts of material.

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In general, a screening assay of the invention makes use of the cells expressing receptor proteins (described above), various heterodimeric receptor ligands, and a candidate compound for testing.

The present invention contemplates screens for small molecule  
5 compounds, including peptides and peptidomimetics, and including receptor ligand analogs and mimics, as well as screens for natural compounds that bind to and agonize or antagonize heterodimeric opioid receptors *in vivo*. Such agonists or antagonists may, for example, interfere in the phosphorylation or dephosphorylation of signal transduction proteins. For example, natural products libraries can be  
10 screened using assays of the invention for such molecules. As used herein, the term "compound" refers to any molecule or complex of more than one molecule that modulates heterodimeric opioid receptor function. The present invention contemplates screens for synthetic small molecule agents, chemical compounds, chemical complexes, and salts thereof as well as screens for natural products, such as  
15 plant extracts or materials obtained from fermentation broths. Other molecules that can be identified using the screens of the invention include opioids, opiates, narcotics, proteins and peptide fragments, peptides, nucleic acids and oligonucleotides, carbohydrates, phospholipids and other lipid derivatives, steroids and steroid derivatives, prostaglandins and related arachadonic acid derivatives, etc.

20 One approach to identifying such compounds uses recombinant bacteriophage to produce large libraries. Using the "phage method" (Scott and Smith, Science, 249:386-390, 1990; Cwirla, *et al.*, Proc. Natl. Acad. Sci.USA, 87:6378-6382, 1990; Devlin *et al.*, Science, 49:404-406, 1990), very large libraries can be constructed ( $10^6$ - $10^8$  chemical entities). A second approach uses primarily chemical  
25 methods, of which the Geysen method (Geysen *et al.*, Molecular Immunology, 23:709-715, 1986; Geysen *et al.*, J. Immunologic Method, 102:259-274, 1987) and the method of Fodor *et al.* (Science, 251:767-773, 1991) are examples. Furka *et al.* (14th International Congress of Biochemistry, Volume 5, Abstract FR:013, 1988; Furka, Int. J. Peptide Protein Res., 37:487-493, 1991) and U.S. Patent Nos. 4,631,211

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and 5,010,175 describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic combinatorial libraries (Needels *et al.*, Proc. Natl. Acad. Sci. USA, 90:10700-4, 1993; Ohlmeyer *et al.*, Proc. Natl. Acad. Sci. USA, 90:10922-10926, 1993; PCT Publication Nos. WO 92/00252 and WO 94/28028) and the like can be used to screen for compounds according to the present invention.

Test compounds may be screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, *e.g.*, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle *et al.*, Tib Tech, 14:60, 1996).

In one embodiment, test compounds are peptides or peptidomimetic compounds generated by rational drug design based on the structure of known opioid peptides, or derived from combinatorial libraries. The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunits may be linked by other bonds, *e.g.*, ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. Thus, peptides of the invention may comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" amino acids (*e.g.*,  $\beta$ -methyl amino acids,  $\alpha$ -methyl amino acids, and  $N\alpha$ -

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methyl amino acids, etc.) to convey special properties to peptides in the library. Additionally, by assigning specific amino acids at specific coupling steps, peptide libraries with  $\alpha$ -helices,  $\beta$ -turns,  $\beta$ -sheets,  $\gamma$ -turns, and cyclic peptides can be generated. A peptide of three or more amino acids is commonly called an  
5 oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

The following non-classical amino acids may be incorporated in peptides of the invention to introduce particular conformational motifs: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Kazmierski *et al.*, 1991, J. Am. Chem. Soc.,  
10 113:2275-2283); (2S,3S)-methyl-phenylalanine, (2S,3R)-methyl-phenylalanine, (2R,3S)-methyl-phenylalanine and (2R,3R)-methyl-phenylalanine; 2-aminotetrahydronaphthalene-2-carboxylic acid (Landis, 1989, Ph.D. Thesis, University of Arizona); hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Miyake *et al.*, 1989, J. Takeda Res. Labs, 43:53-76); b-carboline (D and L)  
15 (Kazmierski, 1988, Ph.D. Thesis, University of Arizona); histidine isoquinoline carboxylic acid, HIC (Zechel *et al.*, 1991, Int. J. Pep. Protein Res., 38:131-138); and histidine cyclic urea (Dharanipragada). The following amino acid analogs and peptidomimetics may be incorporated to induce or favor specific secondary structures: LL-Acp (LL-3-amino-2-propenidone-6-carboxylic acid), a  $\beta$ -turn inducing dipeptide  
20 analog (Kemp *et al.*, 1985, J. Org. Chem., 50:5834-5838);  $\beta$ -sheet inducing analogs (Kemp *et al.*, 1988, Tetrahedron Lett. 29:5081-5082);  $\beta$ -turn inducing analogs (Kemp *et al.*, 1988, Tetrahedron Lett., 29:5057-5060);  $\mu$ -helix inducing analogs (Kemp *et al.*, 1988, Tetrahedron Lett., 29:4935-4938);  $\gamma$ -turn inducing analogs (Kemp *et al.*, 1989, J. Org. Chem., 54:109:115); and analogs provided by the following references: Nagai  
25 and Sato, 1985, Tetrahedron Lett., 26:647-650; DiMaio *et al.*, 1989, J. Chem. Soc. Perkin Trans., p. 1687; also a Gly-Ala turn analog (Kahn *et al.*, 1989, Tetrahedron Lett., 30:2317); amide bond isostere (Jones *et al.*, 1988, Tetrahedron Lett., 29:3853-3856); tretrazol (Zabrocki *et al.*, 1988, J. Am. Chem. Soc., 110:5875-5880); DTC (Samanen *et al.*, 1990, Int. J. Protein Pep. Res., 35:501:509); and analogs taught in

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Olson *et al.*, 1990, J. Am. Chem. Sci., 112:323-333 and Garvey *et al.*, 1990, J. Org. Chem., 56:436.

The coupling of the amino acids may be accomplished by techniques familiar to those in the art (Stewart and Young, 1984, *Solid Phase Synthesis*, Second Edition, Pierce Chemical Co., Rockford, IL; Fields and Noble, 1990, Int. J. Pept. Protein Res., 35:161-214), or using automated synthesizers, such as sold by ABS.

Intact cells expressing heterodimer of interest can be used in screening methods to identify candidate drugs. In one series of embodiments, a permanent cell line is established, *e.g.*, as exemplified below. Alternatively, cells (including without limitation mammalian, insect, yeast, or bacterial cells) are transiently programmed to express the receptor subunit genes by introduction of appropriate DNA or mRNA. Identification of candidate compounds can be achieved using any suitable assay, including without limitation (i) assays that measure selective binding of test compounds; (ii) assays that measure the ability of a test compound to modify (*i.e.*, inhibit or enhance) a measurable activity or function of the receptor; (iii) assays that monitor receptor trafficking, *e.g.*, internalization and re-expression; and (iv) assays that measure the ability of a compound to modify (*i.e.*, inhibit or enhance) transcription of proteins induced by the receptor.

#### **High-Throughput Screen**

Agents according to the invention may be identified by screening in high-throughput assays, including without limitation cell-based or cell-free assays. It will be appreciated by those skilled in the art that different types of assays can be used to detect different types of agents. Several methods of automated assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period of time (*e.g.*, using a 96-well format). For literature references *see, e.g.*, Beggs *et al.*, 1999, J. Biol. Screening, Vol.4, No.3; Renate de Wit *et al.*, 1998, J. Biol. Screening, Vol.3, No.4; Fox *et al.*, 1999, J. Biol. Screening, Vol.4, No.4; Boyd *et al.*, 1996, Clin.Chem., 42:1901-10; Broach *et al.*, 1996, Nature, 384, Supp.:14-16; Cusack *et al.*, 1993, J. Rec. Res., 13:123-134; US Patent Nos. 4,980,281 and 5,876,951; PCT Publication Nos. WO 97/45730, WO 97/14812, and WO 97/10502.

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Such high-throughput screening methods are particularly preferred. The use of high-throughput screening assays to test for agents is greatly facilitated by the availability of large amounts of purified polypeptides, as provided by the invention.

#### Specific Screening Methods

5           There are several screening methods available for the discovery of specific heterodimer receptor ligands. These screens include radioligand binding, signal transduction, expression, reporter assays, and structure function of existing agonists and antagonists. The utilization of yeast as a screening tool can accelerate the search for novel opioid analogs. This technology can be utilized for screening of novel  
10       compounds that are identified in high throughput screens.

#### Radioligand Binding Assays

          Radioligand binding assays allow further characterization of hits from high throughput screens as well as analogs of heterodimer receptor agonists and antagonists. Any of the ligands described below can be radiolabelled for direct binding assays, or  
15       alternatively, used in competitive binding assays.

#### Signal Transduction Assays

          G protein coupled receptors (GPCR) are coupled to a variety of heterotrimeric G proteins, which are comprised of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. Upon agonist binding to a GPCR at the cell surface, conformational changes occur within the  
20       agonist:GPCR complex, which lead to the dissociation of the G protein  $\alpha$  subunit from the  $\beta\gamma$  subunits. The  $G_\alpha$  and  $G_{\beta\gamma}$  subunits then stimulate a variety of intracellular effectors, which transduce the extracellular signal to the inside of the cell. Various signal transduction systems known to be coupled to GPCRs include adenylate cyclase, phospholipase C, phospholipase  $A_2$ , sodium/hydrogen exchange, etc. Thus,  
25       measurements of intracellular calcium concentrations and adenylate cyclase activity indicate whether a hit or test compound is functionally behaving as an agonist or antagonist at the heterodimeric receptor of the invention.

          In a specific embodiment, G-protein signal transduction is coupled to expression of a reporter gene, thus permitting a reporter gene screening assay.

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### Calcium Mobilization Assay

Whole cells expressing the heterodimeric receptor are loaded with a fluorescent dye that chelates calcium ions, such as FURA-2. Upon addition of receptor agonist to these cells, calcium is released from the intracellular stores. The dye chelates these calcium ions. The spectrophotometrically determined ratio of the dye:calcium complexes to free dye provides a numerical measurement of the changes in intracellular calcium concentrations upon addition of opioid-like substrate. Hits from screens and other test compounds can be similarly tested in this assay to functionally characterize them as agonists or antagonists. Increases in intracellular calcium concentrations are expected for compounds with agonist activity while compounds with antagonist activity are expected to block opioid-like substrate stimulated increases in intracellular calcium concentrations.

### Cyclic AMP Accumulation Assay

Upon agonist binding,  $G_s$ -coupled GPCRs stimulate adenylylase. Adenylylase catalyzes the production of cyclic AMP (cAMP) from adenosine-5'-triphosphate which, in turn, activates protein kinases.  $G_i$ -coupled GPCRs are also coupled to adenylylase, however, agonist binding to these receptors results in the inhibition of adenylylase and the subsequent inhibition of cAMP accumulation. To measure the inhibition of cAMP accumulation, cells expressing  $G_i$ -coupled receptors must first be stimulated to elevate cAMP levels. This is achieved by treating the cells with forskolin, a diterpene that directly stimulates cAMP production. Co-incubation of cells expressing  $G_i$ -coupled receptors with forskolin and a functional agonist will result in the inhibition of forskolin-stimulated cAMP accumulation. For a cAMP assay, cells stably expressing a heterodimeric receptor of the invention can be incubated with a test compound, and with forskolin plus a test compound. The cells are then lysed and cAMP levels are measured using the [ $^{125}$ I]cAMP radioimmunoassay (RIA).

### Methods for Detecting Signals

The present invention provides numerous methods for detecting signals, including but not limited to directly detecting phosphorylation of proteins using

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radioactive phosphorous compounds, indirectly detecting phosphorylation with antibodies specific for phosphorylated epitopes, or detecting signals from activated signal transduction proteins, such as gene expression. Preferably, gene expression is detected using a reporter gene assay. Alternatively, a downstream element of a signal transduction pathway can be modified to have reporter activity, *i.e.*, the reporter gene can be activated by signals generated as a consequence of receptor binding, rather than as a direct result of receptor binding. Reporter genes for use in the invention encode detectable proteins, including, but are by no means limited to, chloramphenicol transferase (CAT),  $\beta$ -galactosidase ( $\beta$ -gal), luciferase, green fluorescent protein (GFP), yellow fluorescent protein (YFP), alkaline phosphatase, and other genes that can be detected, *e.g.*, immunologically (by antibody assay).

In one embodiment, the instant invention discloses the use of the Bioluminescence Resonance Energy Transfer (BRET) method for the detection of protein-protein interactions (*see, e.g.*, Xu *et al.*, Proc. Natl. Acad. Sci. U S A, 96:151-156, 1999; Angers *et al.*, Proc. Natl. Acad. Sci. U S A, 97:3684-3689, 2000). BRET measures the transfer of energy between a luminescent donor (*e.g.*, luciferase expressed as a fusion protein with one of the receptors) and a fluorescent acceptor (*e.g.*, YFP expressed as a fusion protein with one of the receptors). According to the present invention, BRET can be used (i) to examine the heterodimerization between various receptors (*i.e.*, opioid/opioid receptors and opioid/catecholamine receptors) and (ii) to study the effect of agonist/antagonist binding on the level of dimers (*see* Example 4, *infra*).

In another embodiment, a yeast screening assay, useful for testing agonists and antagonists of mammalian G-protein coupled receptors, *e.g.*, as disclosed in U.S. Patent No. 5,482,832, can be used.

Specific but not limiting examples of opioid receptor-evoked cellular responses that can be monitored are summarized in Table 1.

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**Table 1 - Opioid Receptor-Evoked Cellular Responses****Direct G-protein  $\beta\gamma$  or  $\alpha$  subunit-mediated effects**

- activation of an inwardly rectifying potassium channel
- inhibition of voltage operated calcium channels (N, P, Q, and R type)
- 5 inhibition of adenylyl cyclase

**Responses of unknown intermediate mechanism**

- activation of  $PLA_2$
- activation of  $PLC\beta$  (possibly direct G protein  $\beta\gamma$  subunit activation)
- 10 activation of MAPKinase
- activation of large conductance calcium-activated potassium channels
- activation of L type voltage operated calcium channels
- inhibition of T type voltage operated calcium channels
- direct inhibition of transmitter exocytosis
- 15

**Responses which are a consequence of opioid-evoked changes in other effector pathways**

- activation of voltage-sensitive potassium channels (activation of  $PLA_2$ )
- inhibition of M channels (activation of  $PLA_2$ )
- 20 inhibition of the hyperpolarisation-activated cation channel ( $I_h$ ) (Reduction in cAMP levels following inhibition of adenylyl cyclase)
- elevation of intracellular free calcium levels (activation of  $PLC\beta$ , activation of L type voltage operated calcium conductance)
- potentiation of NMDA currents (activation of protein kinase C)
- 25 inhibition of transmitter release (inhibition of adenylyl cyclase, activation of potassium channels and inhibition of voltage operated calcium channels)
- decreases in neuronal excitability (activation of potassium channels)
- increases in neuronal firing rate (inhibition of inhibitory transmitter release-disinhibition)

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changes in gene expression (long-term changes in adenylyl cyclase activity, elevation of intracellular calcium levels, activation of cAMP response element binding protein (CREB))

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### **Opioid Receptor Ligands**

Various opioid receptor ligands, both synthetic molecules and endogenous opioid peptides, are known in the art (*see, e.g.*, Tocris product literature) and are commercially available, *e.g.*, from Tocris Cookson Inc. (USA). Tables 2 and 3 summarize some of such ligands, but are not intended to be limiting. These can serve as prototypes for drugs or drug design, based on targeting each subunit of the heterodimeric receptors of the invention. In particular, the invention contemplates designing heterodimeric receptors by linking subunit-specific ligands to each other using routine coupling chemistry.

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**Table 2 - Opioid Ligands**

Receptor Type	$\mu$ -Receptor	$\delta$ -Receptor	$\kappa$ -Receptor	ORL <sub>1</sub>
Selective agonists	endomorphin-1 endomorphin-2 DAMGO	[D-Ala <sup>2</sup> ]-deltorphan I [D-Ala <sup>2</sup> ]-deltorphan II DPDPE DSLET SNC80 SNC121	enadoline BRL-52537 ICI-199,441 ICI-204,448 N-Methyl-N-[(1S)-1-phenyl-2(1-pyrrolidinyl)-ethyl]phenylacetamide ( $\pm$ )-1-(4-Trifluoromethylphenyl)acetyl-2-(1-pyrrolidinyl)methylpiperidine ( $\pm$ )-U-50488 (+)-U-50488 (-)-U-50488 U-54494A U-69593	nociceptin / OFQ Ac-RYYRWK-NH <sub>2</sub> * nocistatin Noc II
Selective antagonists	CTOP Clocinnamox Etonitazenyl isothiocyanate $\beta$ -Funaltrexamine Naloxonazine	naltrindole N-benzylnaltrindole naltriben TIPP- $\Psi$ ICI 174864 ICI 154129 BNTX	nor-binaltorphimine DIPPA	None as yet**

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<b>Radioligands</b>	[ <sup>3</sup> H]-DAMGO	[ <sup>3</sup> H]-deltorphan II [ <sup>3</sup> H]-naltrindole [ <sup>3</sup> H]-pCI-DPDPE [ <sup>3</sup> H]-SNC 121	[ <sup>3</sup> H]-enadoline [ <sup>3</sup> H]-U69593	[ <sup>3</sup> H]-nociceptin
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\*Related combinatorial library hits are also selective agonists (Dooley, *et al.*, J. Pharmacol. Exp. Ther., 283:735, 1997).

- 5   \*\*Ac-RYYRIK-NH<sub>2</sub> has been proposed to be an ORL<sub>1</sub> antagonist (Grevel and Sadee, Science, 221:1198, 1983) whereas the putative antagonist [Phe<sup>1</sup>Ψ(CH<sub>2</sub>-NH)Gly<sup>2</sup>]nociceptin (1-13)NH<sub>2</sub> (Guerini *et al.*, Br. J. Pharmacol., 123:163, 1998) appears to be a partial agonist.

**Table 3 - Mammalian Endogenous Opioid Ligands**

10	<b>Precursor</b>	<b>Endogenous Peptide</b>
	Pro-opiomelanocortin	β-Endorphin
	Pro-enkephalin	[Met]enkephalin [Leu]enkephalin
		Metorphamide
	Pro-dynorphin	Dynorphin A Dynorphin A(1-8) Dynorphin B α-neoendorphin β-neoendorphin
15	Pro-nociceptin / OFQ	Nociceptin
	Not known yet*	Endomorphin-1 Endomorphin-2

- 20                   In addition to the opioid receptor ligands discussed above, various other GPRC ligands particularly catecholamines, can be used to prepare bi-functional, bi-specific compounds by joining them to an opioid receptor ligand, in connection with the present invention. Examples of some ligands are described in Table 4; additional ligands can be found, *inter alia*, in product literature and catalogs from
- 25   Tocris (<http://www.tocris.com>).